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Detection of GB Virus-C/Hepatitis G Virus RNA in Serum by Reverse Transcription Polymerase Chain Reaction

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Three PCR methods based on the GB virus-C/hepatitis G virus (GBV-C/HGV) 5′UTR and NS3 genomic region were used for the detection of GBV-C/HGV RNA in serum of 62 patients with chronic hepatitis C virus (HCV) infection. Ten of 62 (16%) patients were found to have GBV-C/HGV RNA, which was confirmed by sequence analysis of the 5′UTR PCR amplicon. All methods appear to be specific, but methods based on the 5′UTR appear to be more sensitive. ***J. Med. Virol. 52:92–96, 1997.*** © 1997 Wiley-Liss, Inc.

KEY WORDS: GBV-C; HGV; HCV; RNA; PCR; hybridization; serum; sensitivity; specificity

INTRODUCTION

Recently, research groups at Abbott Laboratories and Genelabs discovered independently a new hepatitis virus which they have named GB virus C (GBV-C) and hepatitis G virus (HGV), respectively [Simmons et al., 1995a; Leary et al., 1996a; Linnen et al., 1996]. Two other GB viruses, GBV-A and GBV-B, which were believed to be novel flavivirus-like agents in tarmarins, were also discovered and were believed to be animal viruses that are related to GBV-C and HGV [Simmons et al., 1995b; Muerhoff et al., 1996]. The nucleotide sequences and predicted amino acid sequences between GBV-C and HGV showed good homology, and hence these probably represent isolates from the same group of viruses. For the sake of simplicity, we shall refer to these viruses as GBV-C/HGV in this paper [Zuckerman, 1996].

Studies of blood product recipients have demonstrated the transmissibility of GBV-C/HGV [Linnen et al., 1996]. However, whether this infective agent GBV-C/HGV is a hepatitis virus or not is still a subject of debate, since no firm association between this infective agent and liver disease has been documented. At present, there is no commercially available serological assay, and most investigators study the prevalence of GBV-C/HGV by utilizing sensitive molecular tools like reverse transcription polymerase chain reaction (RT-PCR) [Simons et al., 1995a; Linnen et al., 1996; Leary et al., 1996b].

GBV-C/HGV has a positive-sense, single-stranded RNA genome of approximately 9.4 kb and is a member of the Flaviviridae family. Phylogenetically, GBV-C/HGV is closely related to HCV [Simons et al., 1995; Linnen et al., 1996]. The genomic organization of GBV-C/HGV is also similar to hepatitis C virus (HCV) with a 5′untranslated region (UTR), a single open reading frame with the structural proteins on the 5′end and nonstructural (NS) proteins at the 3′end, and a 3′UTR. Recent studies have shown that HGV infection is common among patients with chronic HCV infection [Linnen et al., 1996; Berenguer et al., 1995]. Hence a sensitive technique for the specific detection of GBV-C/HGV without cross reactivity to HCV is essential in order to study the epidemiology of HGV infection. The aim of the present study was to develop a sensitive RT-PCR based assay for the sensitive and specific detection of GBV-C/HGV RNA in serum.

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TABLE I. Primers and Probes Used in This Study

Primers		Expected product size
5′UTR RT-"nested" PCR		
Outer primers		
Antisense	5′-ATG CCA CCC GCC CTC ACC CGA A-3′	
Sense	5′-AAA GGT GGT GGA TGG GTG ATG-3′	425 bp
Inner primers		
Antisense	5′-CCC CAC TGG TCY TTG YCA ACT C-3″	
Sense	5′-AAT CCC GGT CAY CYT GGT AGC CAC T-3″	253 bp
5′UTR RT-PCR and radiolabeled probe hybridization		
Primers		
Antisense	5′-CCC CAC TGG TCY TTG YCA ACT C-3″	
Sense	5′-AAT CCC GGT CAY CYT GGT AGC CAC T-3″	253 bp
Probe	5′-GTA CGG TCC ACG TCG CCC TTC AAT G-3″	
NS3 RT-PCR and radiolabeled probe hybridization		
Primers		
Anti-sense	5′-ACN ACN AGG TCN CCR TCY TTG ATG AT-3′	
Sense	5′-GGN RMK RTY CCY TTT TAT GGG CAT GG-3′	188 bp
Probe GBV-C sequence probe		(Abbott Laboratories)

Y = C or T; N = A or T or C or G; R = A or G; M = A or C; K = G or T.

MATERIALS AND METHODS

Sixty-two patients with chronic HCV infection who attended the liver clinics at Shands Health Center at the University of Florida Health Science Center were studied (M:F, 37:25; mean age, 51 years; range, 30–72). All patients were seropositive for antibody to HCV by second-generation enzyme immunoassay (Ortho Diagnostics, Raritan, NJ, or Abbott Laboratories, North Chicago, IL) and HCV RNA by RT-"nested" PCR using primers derived from the highly conserved 5′UTR as previously described [Lau et al., 1993]. All had elevated serum alanine aminotransferase (ALT) levels for at least 6 months and were seronegative for hepatitis B surface antigen and human immunodeficiency virus markers. A thorough history and physical examination and appropriate laboratory tests ruled out other causes of liver disease.

All known GBV-C and HGV sequences were obtained from the Genbank and maximally aligned. Two genomic regions were studied as potential target regions for developing PCR-based assays, namely, the relatively well-conserved 5′UTR and NS3 region. The latter has recently been reported [Leary et al., 1996]. Three prerequisites were essential for the design of the primers from the 5′UTR region: first, conserved regions between the GBV-C and HGV isolates; second, the sequences needed to lack significant homology with any of the known HCV, pestiviruses, and flaviviruses sequences; and finally, the sequences needed to have molecular characteristics from which to design good primers, such as nucleotide composition, T_m, and length. The primers utilized for the NS3-based assay were identical to those previously reported [Leary et al., 1996] (Table I).

Extraction of RNA From Serum

Serum specimens were spun and separated within 4 hours after venesection and stored at −20°C. Sera were thawed only once or twice on ice before this study. One hundred μl of serum was pipetted into 450 μl of Chomczynski denaturing solution D containing 20 mg/ml bromophenol blue in a sterile screw-capped 2 ml tube [Chomczynski et al., 1987; Lin et al., 1992]. Fifty μl of acetate buffer (pH 4), 500 μl acidic phenol (pH 4.5, Amresco, Solon, OH), and 125 μl of chloroform-isoamyl alcohol (ratio 24:1; Amresco) were added and the mixture was shaken for 30 minutes at room temperature. After 15 minutes of centrifugation (12,000g), 400 μl of the aqueous phase (two-thirds of the aqueous phase to avoid organic solvent contamination during pipetting) was transferred to a sterile 2 ml polypropylene tube containing 40 μl of precipitation mixture (2.6 M sodium acetate, pH 6.5, glycogen 1.25 g/L) and 1 ml of absolute ethanol (200 proof). The solution was mixed and stored at −70°C overnight. The tubes were centrifuged (4°C, 12,000g) for 45 minutes. The supernatant fluid was discarded, 1 ml of 75% ethanol (in DEPC treated water) was added to the tube without disturbing the pellet, and the mixture was centrifuged for another 5 minutes. The supernatant was carefully discarded and moisture was removed with a sterile cotton tip. The pellet was dissolved in 13 μl of sterile PCR buffer (0.01 M Tris, pH 8.3, 0.05 M KCl) containing 0.5% Nonidet P-40 and bromophenol blue (20 mg/L) to which RNase inhibitor (0.05% U/μl, Promega, Madison, WI) and dithiothreitol (0.005 M) were freshly added. After addition of 30 μl mineral oil, the tube was incubated at 37°C for 15 minutes and then briefly centrifuged. This RNA preparation was used for RT-PCR testing as described below.

RT-PCR for the Detection of GBV-C/HGV RNA

Three different RT-PCR protocols were tested: (1) RT- "nested" PCR based on 2 sets of primers designed from GBV-C/HGV 5′UTR; (2) RT and single round PCR based on 5′UTR with detection of the amplicon by radiolabeled probe hybridization; and (3) NS3-based RT-PCR as described by Leary et al. Four μl of the prepared RNA was used for the generation of cDNA by

random priming using Moloney murine leukemia virus (MMLV) reverse transcriptase (final concentration 0.5 U/μl, Gibco BRL, Gaithersburg, MD) in a 20 μl volume. Ten μl of the synthesized cDNA (equivalent to RNA extracted from 10 μl of serum) was used for the PCR reactions.

For the RT-"nested" PCR method based on 5′UTR, the cDNA was amplified with the outer primers (Table I) in a 25 μl volume containing 0.01 M Tris buffer (pH 8.3), 0.05 M KCl, 0.25 mM of each of 4 dNTPs, 1.8 mM $MgCl_2$, 1.0 μM of each of the primers, and Taq DNA polymerase (0.03 U/μl, Promega). The tube was briefly centrifuged and put onto a DNA thermal cycler (9600, Perkin-Elmer Cetus, CA) at 94°C for 4 minutes. This was followed by 40 cycles of 94°C (denaturing), 55°C (annealing), and 72°C (extension) for 1 minute each, and finally 72°C for 5 minutes. Two μl of the first round PCR mixture was added to the second round PCR mixture containing the same buffer, 0.2 mM of each dNTPs, 1.8 mM $MgCl_2$, 2.0 μmol/L each of the internal primers, and Taq DNA polymerase (0.03 U/μl, Promega), centrifuged briefly, and subjected to another round of PCR as in the first round of PCR but with a 5-minute incubation at 94°C prior to cycling. Eight μl of the PCR product was then mixed with 3 μl of the loading buffer, electrophoresed in 4% NuSieve 3:1 agarose with ethidium bromide in the buffer and gel, and visualized under ultraviolet illumination.

For the second method, PCR was carried out for one round amplifying the 5′UTR using the inner primers (as described above), in a 25 μl volume using the same conditions as described above for PCR using outer primers. The PCR amplicons were then applied onto a presoaked positively charged nylon membrane (Boehringer Mannheim, Indianapolis, IN) using a manifold apparatus (Gibco). Eight μl of the PCR product was added to a mixture containing NaOH 0.4M and EDTA 10 mM in distilled water, which was then applied to each well under continuous suction. The wells were then rinsed with 100 μl of 0.4 M NaOH, the membrane was then equilibrated with 2 × SSC, and the DNA was cross-linked to the membrane in a ultraviolet chamber with 30 mJ (BioRad, Hercules, CA). The membrane was prehybridized in a solution containing 10 × SSC, 1% SDS, 5× Denhardt's solution, 0.1 M $NaPO_4$, pH 7.0, and 10 μg Herring testes DNA at 42°C for 2–3 hours. The oligonucleotide probe (Table II) was end-labeled with gamma ^{32}P-ATP (specific activity 50 μCi/μl, New England Nuclear, Boston, MA) with 10 U of polynucleotide kinase in a 20 μl volume at 37°C for 90 minutes. The labeled probe was purified with a Sephadex G25 spin column to remove unincorporated ATP and added into 10 ml of prehybridization solution without Herring testis DNA for the hybridization of the membrane at 42°C for 4 hours. The blot was then washed successively at 2 × SSC, then 0.1×SSC/0.1% SDS, and finally 2 × SSC with Geiger monitoring for the background. The blot was then exposed to a film using an intensifier at −70°C.

The amplification of the NS3 and radiolabeling was essentially the same as recommended previously [Leary et al., 1996]. Briefly, total RNA from 10 μl serum equivalent (instead of 5 μl equivalent in the original paper) was used for RT-PCR. PCR was performed by touchdown PCR for a total 53 cycles (43 cycles: 94°C for 20 seconds, 55°C for 30 seconds, decreasing by 0.3°C per cycle, 72°C for 60 seconds; followed by 10 cycles: 94°C for 20 seconds, 40°C for 30 seconds, 72°C for 60 seconds) and terminated with incubation at 72°C for 10 minutes as recommended (Mg^{2+} concentration 1.25 mM). The PCR product was analyzed by agarose gel electrophoresis, stained with ethidium bromide, and visualized under ultraviolet illumination. In addition, the PCR products were also spot-blotted on a nylon membrane using a Manifold apparatus instead of Southern blotting to a nylon membrane as originally described [Leary et al., 1996], cross-linked, and prehybridized as above. The DNA probe kindly provided by Abbott Laboratories (lot no. 07645F9) was labeled with alpha ^{32}P-dCTP (50 μCi/μl, New England Nuclear) using a Rediprime labeling kit (Amersham, Arlington Heights, IL) according to the manufacturer's instructions. The labeled probe was purified with a G50 column. The hybridization was overnight, washings were achieved with 2×SSC/0.1% SDS and 1×SSC/0.1% SDS, respectively, at 65°C.

TABLE II. Comparison of the Three Methods for the Detection of GBV-C/HGV RNA in Serum

		5′UTR RT-"nested" PCR	
		Positive	Negative
5′UTR RT-PCR only	positive	6	0
	negative	4	52
5′UTR RT-PCR and radioprobing	positive	10	0
	negative	0	52
		5′UTR RT-"nested" PCR	
		Positive	Negative
NS3 RT-PCR only	positive	7	0
	negative	3	52
NS3 RT-PCR and radioprobing	positive	8	0
	negative	2	52

Nucleotide Sequence Analyses

To confirm that our RT-PCR assay was specific, the amplicon from all patients who tested positive for GBV-C/HGV RNA based on the RT-"nested" PCR assay were sequenced directionally by Taq cycle sequencing (Epicenter Technologies, Madison, WI) according to the manufacturer's instructions. Direct sequence comparison was performed to confirm their identity.

Preparation of HGV RNA Transcripts

To determine the sensitivity of our nested PCR, synthetic RNA transcripts were generated using a previously described strategy [Collins et al., 1995]. A plasmid (T27036, Genelabs) containing the HGV 5′UTR under the control of a SP6 promoter in pCR™ II vector (Invitrogen, San Diego, CA) was linearized by Xbal digestion. The in vitro transcript was prepared from 5 μg

of the linearized plasmid using the MEGAscript in vitro Transcription kit (Ambion, Austin, TX). α^{32}P-GTP was added as a tracer to the transcription reactions. The quality of the transcript was characterized by electrophoresis on a 1.5% formaldehyde gel which was dried and scanned on an Ambis 4000 Radioanalytic Imager (AMBIS, Inc., San Diego, CA). DE81 chromatography was performed to verify purity. The HGV transcript was greater than 85% full length and contained less than 5% free nucleotides. Absorbance at 260 (A260) was used to quantify the transcript. Serial dilutions of the RNA were then used for the determination of the sensitivity of the RT-"nested" PCR assay based on the 5′UTR region.

RESULTS

A comparison of the detection by the three protocols is shown in Table II. RT-"nested" PCR based on the 5′UTR genomic region detected GBV-C/HGV RNA in 10 of the 62 patients with chronic HCV infection. RT and a single round of PCR based on the 5′UTR revealed PCR amplicon of the correct size as detected by ethidium bromide staining and visualized under ultraviolet light in 6 of the 10 patients. When radiolabeled hybridization was applied, specific PCR amplicons were detected in all 10 patients positive by RT-"nested" PCR. GBV-C/HGV RNA was detected by RT with a single round of NS3-based PCR in 7 patients with gel electrophoresis analysis and 8 with radiolabeled hybridization. There were 2 patients who were positive for GBV-C/HGV RNA by 5′UTR-based RT-PCR but not by the NS3-based method.

The inner PCR amplicons from all 10 HGV RNA positive patients were sequenced. Sequence comparison confirmed that these sequences were related to GBV-C/HGV (Fig. 1). These sequences were also confirmed not related to HCV, flavi-, or pestiviruses. These data confirm the specificity of these RT-PCR assays, and demonstrate the specificity of the tests in HCV and GBV-C/HGV coinfected patients.

Since the RT-"nested" PCR is more convenient and does not involve the use of radioactive labels, this assay was further studied in order to determine its detection limit. Using serial dilutions of the HGV RNA transcripts with known copy number, it was shown that the RT-"nested" PCR assay consistently detected down to 30 copies of HGV RNA per sample (and commonly less than 20 copies). Thus, assuming that the extraction efficiency of HGV RNA is 100%, the detection limit of the RT-"nested" PCR assay is consistently less than 3,000 HGV genome equivalent/ml.

DISCUSSION

This report compares 3 methods for the detection of GBV-C/HGV RNA based on RT-PCR in either the 5′UTR or NS3 genomic region. The detection of GBV-C/HGV RNA by 5′UTR appears to be both sensitive, as confirmed by quantitation utilizing RNA transcript standardization, and specific, as documented by sequence comparison and phylogenetic tree analysis. The RT-"nested" PCR approach appears to be an attractive technique, since it does not involve the use of radioactive label. The NS3-based method was also highly spe-

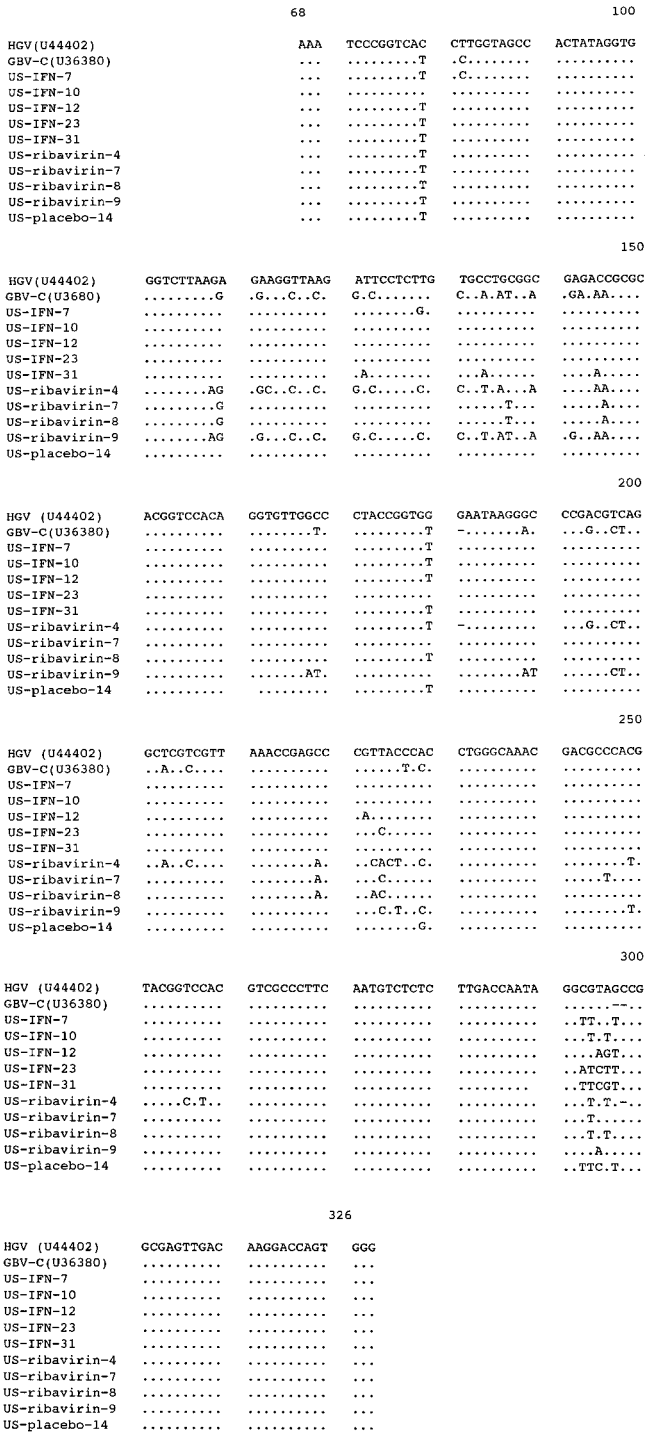

Fig. 1. Alignment of the 5′UTR sequences of the 10 US GBV-C/HGV isolates with HGV (Genbank accession number U44402) and GBV-C (accession number U36380). The numbering system is according to the HGV sequence. "–" indicates deletion in that particular nucleotide position.

cific but was less sensitive than the assays based on 5′UTR. This may be related to greater genetic heterogeneity in the NS3 region.

In designing our assays, we elected to dissolve the RNA in a large volume and use only a fraction of the RNA for cDNA synthesis. In addition, only half of the cDNA was used for PCR. This design allowed us to retest the results, if necessary. If these steps were deleted, i.e., the extracted RNA was dissolved in a small volume and the entire cDNA was used for RT-PCR, the detection limit would be projected to be as low as 500 genome equivalents/ml.

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